A Prototypic Lysine Methyltransferase 4 from Archaea with Degenerate Sequence Specificity Methylates Chromatin Proteins Sul7d and Cren7 in Different Patterns*[S]

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Background: The origin of eukaryotic histone modification enzymes still remains obscure.
Results: Prototypic KMT4/Dot1 from Archaea targets chromatin proteins (Sul7d and Cren7) and shows increased activity on Sul7d, but not Cren7, in the presence of DNA.
Conclusion: Promiscuous aKMT4 could be regulated by chromatin environment.
Significance: This study supports the prokaryotic origin model of eukaryotic histone methyltransferases and sheds light on chromatin dynamics in Archaea.

Histone methylation is one of the major epigenetic modifications even in early diverging unicellular eukaryotes. We show that a widespread lysine methyltransferase from Archaea (aKMT4), bears striking structural and functional resemblance to the core of distantly related eukaryotic KMT/Dot1. aKMT4 methylates a set of various proteins, including the chromatin proteins Sul7d and Cren7, and RNA exosome components. Csl4- and Rrp4-exosome complexes are methylated in different patterns. aKMT4 can self-methylate intramolecularly and compete with other proteins for the methyl group. Automethylation is inhibited by suitable substrates or DNA in a concentration-dependent manner. The automethylated enzyme shows relatively compromised activity. *aKMT4*-8A mutant with abrogated automethylation shows a more than 150% increase in methylation of substrates, suggesting a possible mechanism to regulate methyltransferase activity. More interestingly, methylation of Sul7d, but not Cren7, by aKMT4 is significantly enhanced by DNA. MS/MS and kinetic analysis further suggest that aKMT4 methylates Sul7d in the chromatin context. These data provide a clue to the possible regulation of aKMT4 activity by the local chromatin environment, albeit as a promiscuous enzyme required for extensive and variegated lysine methylation in *Sulfolobus*. This study supports the prokaryotic origin model of eukaryotic histone modification enzymes and sheds light on regulation of archaeal chromatin.

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As the basic building blocks of chromatin, DNA-associated small basic proteins, such as eukaryotic histones, are subject to various reversible covalent modifications that play pivotal roles in regulating chromatin structure, chromatin accessibility, and gene expression (1–3). Lysine methylation is one of the most common types of post-translational modification. In eukaryotes, there are two distinct classes of histone lysine methyltransferases (KMTs),[3] the SET domain and Dot1 families (3). All known KMTs except Dot1 bear a conserved 130-amino acid SET (Su(var)3–9, enhancer of Zeste (E(Z)), and trithorax (Trx)) domain (4). Instead, Dot1 possesses a conserved catalytic core characteristic of class I methyltransferases (MTases) (5, 6). The Dot1 family consists of only one evolutionarily conserved member called Dot1 (also named KMT4) (4). Dot1 is the sole MTase for mono-, di-, and trimethylation of histone H3K79 and can only methylate H3K79, which lies within the histone core, in the context of the nucleosome and cannot methylate free H3 (5, 7–9). It was originally identified as a high copy disruptor of telomeric silencing in *Saccharomyces cerevisiae* (10). Two recent studies have demonstrated that the role of Dot1 in heterochromatin gene silencing might be overestimated in the original *URA3* telomere silencing reporter assays in yeast (11, 12). Nevertheless, Dot1 participates in heterochromatin formation through competing with Sir3 for a binding site on histone H4 (13). In addition, H3K79 methylation could reduce the affinity of SIR complex for chromatin (4, 14). Mammalian Dot1L has been shown to play crucial roles not only in transcription, cell cycle regulation, and DNA damage response, but also in embryogenesis, development, and differentiation (4).

It is still an open question whether epigenetic mechanisms similar to histone methylation also exist in prokaryotes. In Bacteria, there are no reports of covalent modification of nucleoid proteins (HU, IHF, Fis, and H-NS) thus far (15). Archaea, the

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[S] This article contains supplemental Tables S1–S3 and Figs. S1 and S2.
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[3] The abbreviations used are: KMT, lysine methyltransferase; AdoMet, S-adenosylmethionine; [³H]AdoMet, S-[*methyl*-³H]adenosylmethionine; MTase, methyltransferase; H3K79, histone H3 Lys-79.

third domain of life (16), bear some features strikingly similar to Eukarya with respect to transmission of genetic information during DNA replication, repair, and transcription (17, 18). A variety of abundant basic DNA-binding proteins are found to associate with DNA and maintain chromatin structure in Archaea (19). They are proposed to be functional homologs of eukaryotic histones despite the fact that most of them lack significant sequence or structural similarity (20, 21).

One of the main branches of Archaea, Euryarchaeota, wrap their DNA using true histones, which are organized into tetrameric nucleosomes. Archaeal histones share common ancestry with the histone fold domains of eukaryotic H3 and H4 (20). Nevertheless, the archaeal histones lack the N- and C-terminal tails of their eukaryotic counterparts, which contain the principal sites of modification and therefore exhibit no detectable covalent modifications (20, 22). Interestingly, all archaeal chromatin proteins other than histones do seem to undergo a variety of post-translational modifications (19, 22).

The hyperthermophilic *Sulfolobus*, which belongs to the phylum Crenarchaeota, uses architectural proteins, such as Sul7d and Cren7, instead of histones. Among archaeal chromatin proteins, members of the Sul7d family are the most abundant, comprising about 5% of the soluble cellular proteins (19, 23). Sul7d proteins are modified by monomethylation of N- or C- terminal lysine residues to different extents in a strain-dependent manner (23, 24). The number of methylated lysine residues increases *in vivo* with elevated growth temperature (24). It has been proposed that this modification might have a direct or indirect effect on gene expression and thermal adaptation. Supporting this idea, Cren7 is methylated at some of its 12 lysine residues, which may affect the chromatin structure (25).

The enzyme(s) responsible for methylation of crenarchaeal proteins, such as Sul7d and Cren7, have not yet been identified. Manzur and Zhou (26) identified the first SET lysine methyltransferase from Archaea, Gö1-SET, in a few *Methanosarcina* species, which selectively catalyzes the methylation of Lys-37 on DNA-associated protein MC1-α. Here, we report that many Archaea, especially Crenarchaea, encode a lysine methyltransferase, which shows structural and enzymatic similarity to the eukaryotic KMT4/Dot1 family. We designated it as aKMT4 (belonging to the KMT4/Dot1 family of class I AdoMet-dependent MTases). Recently, Chu *et al.* (27) also isolated and characterized the same enzyme from *Sulfolobus* (designated as aKMT by them) and found that it methylates Cren7 and many proteins involved in DNA replication *in vitro*, which correlates well with the extensive lysine methylation pattern in *Sulfolobus* cells (28). Our study shows that aKMT4 bears self-methylation activity and competes for a methyl group with other substrates. Methylation of Sul7d, but not Cren7, by aKMT4 is found to be significantly stimulated by DNA. MS/MS data show that, only when DNA is added, the *in vitro* methylation pattern of Sul7d by aKMT4 correlates with that of native Sul7d. Our data suggest that the activity of aKMT4 might be regulated by the local chromatin environment in *Sulfolobus* cells, albeit as a prototype of the KMT4/Dot1 family with extremely low sequence specificity.

EXPERIMENTAL PROCEDURES

Bioinformatics Analyses—KMT4/Dot1 orthologs were first identified by PSI-BLAST searches using the protein sequence of Dot1 from *Saccharomyces cerevisiae* in the NCBI genome database (29). Archaeal homologs of Dot1-like methyltransferase (*SiRe-1449*) were identified with an *E* value cut-off of 1×10^{-8}. The methyltransferase domain was confirmed by performing a search in CDD (30). All putative Dot1 methyltransferase homologs were extracted and aligned using MUSCLE with default settings (31). The phylogenetic tree was calculated by the neighbor joining method with JTT matrix using MEGA5 (32). The bootstrap values were estimated with 500 replications. The secondary and tertiary structures of archaeal KMT4 protein were generated by comparative modeling based on alignment and the crystal structure of yeast Dot1 (33) using the PSIPRED (34) and I-TASSE (35) programs, respectively. The homology modeling was evaluated by COLORADO3D (36).

Organisms and Culture Conditions—*Sulfolobus islandicus* REY15A strain was kindly provided by Dr. Qunxin She (University of Copenhagen). *Sulfolobus* cells were grown as described earlier (37, 38).

Genomic DNA Isolation, Gene Cloning, and Mutagenesis—Genomic DNA was isolated from *S. islandicus* cells by the phenol/chloroform extraction method (38). The gene encoding aKMT4 (*SiRe-1449*, accession number YP_005648729) in *S. islandicus* was PCR-amplified from genomic DNA and cloned into either pET28a (NcoI and EagI sites) or pGEX-4T-1 (SalI and EagI sites). Point mutations were introduced using the QuikChange site-directed mutagenesis kit from Stratagene. Oligonucleoties used for gene cloning (for sequence, see supplemental Table S1) and mutagenesis (supplemental Table S2) were synthesized by Invitrogen. Plasmids constructed in this study are summarized in supplemental Table S3. They were all verified for the presence of the intended mutations and the absence of additional mutations by sequencing.

Protein Overproduction and Purification—All recombinant proteins were overexpressed in *Escherichia coli* BL21 (DE3) CodonPlus RIL (Stratagene). aKMT4 was purified by heat at 75 °C for 15 min, followed by HiTrap chelating or glutathione-Sepharose columns (GE Healthcare). GST tag was removed by treatment with 3 units of thrombin/mg of fusion protein and concentrated to 4 mg/ml in 50 mM Tris-HCl (pH 7.3), 100 mM NaCl, 1 mM DTT, and 5% glycerol. Sul7d and Cren7 were purified as described (25, 37). Rrp4-exosome and Csl4-exosome complexes were reconstituted using purified subunits as described (39). Mutant proteins were expressed and purified by a procedure identical to the one for their wild-type proteins.

Protein Methylation Reactions—Standard protein methylation reactions (total 30 μl) were set up containing methyltransferase aKMT4 (4 μM) and one of the various substrate proteins (1–12 μM) in the reaction buffer (10 mM Tris-HCl, pH 8.0, 1 mM DTT, 5% glycerol, 1 μl of *S*-[*methyl*-³H]adenosylmethionine ([³H]AdoMet; 15 Ci/mmol; PerkinElmer Life Sciences) except as specifically indicated. All standard reactions were carried out at 50 °C for 3 h and then quenched by boiling for 5 min in SDS-PAGE loading buffer. At the beginning, the methyltransferase activity was measured by a simple approach. The reac-
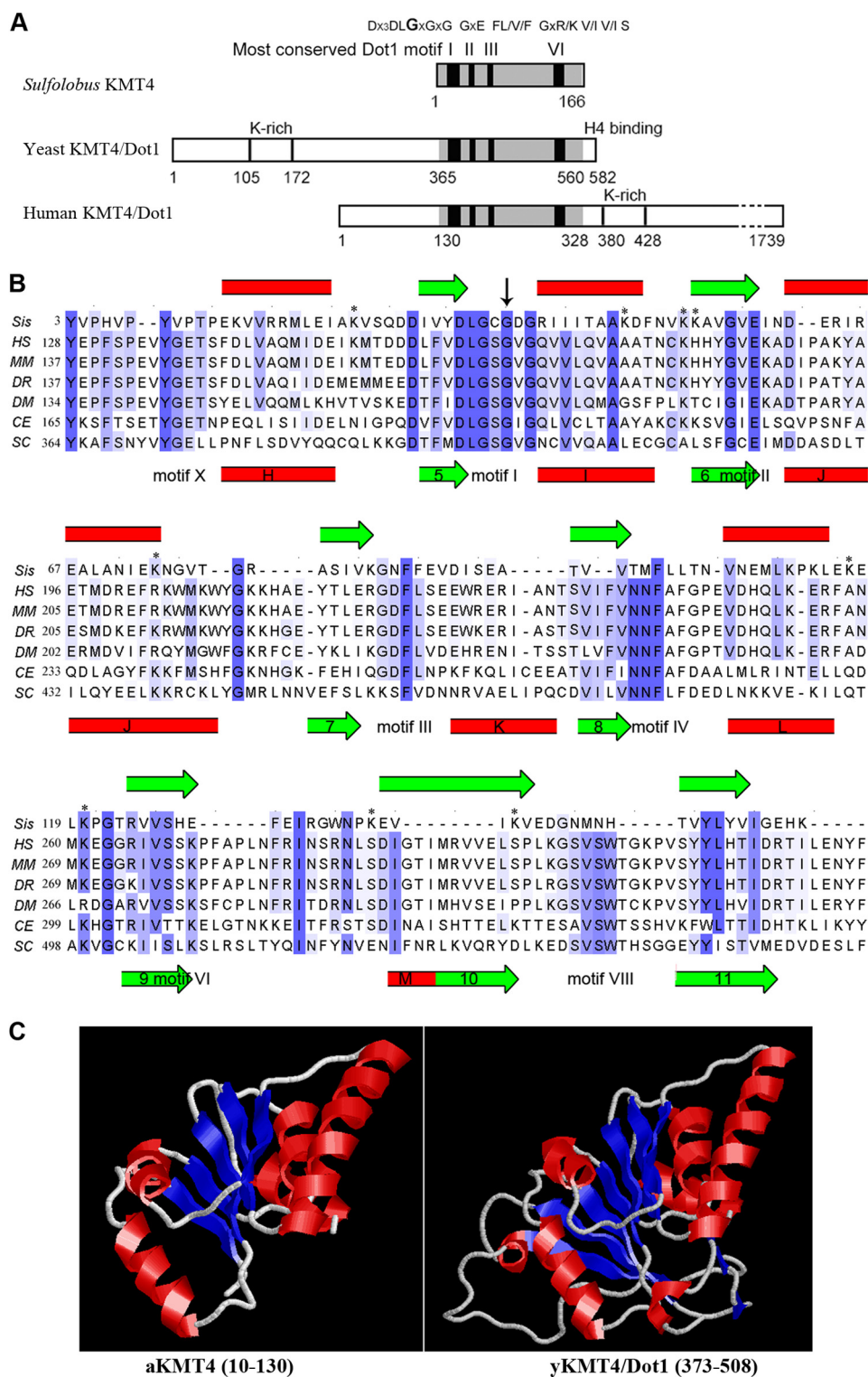
FIGURE 1. **Prototypic KMT4/Dot1 methyltransferases are conserved in Archaea.** *A*, domain organization of KMT4/Dot1 family MTases from *Sulfolobus*, budding yeast, and humans. *B*, structure-based multiple-sequence alignment of aKMT4 and eukaryotic orthologs. The sequences were aligned using MUSCLE with default settings. Individual residues with identity or similarity across the whole alignment are *highlighted* in *blue*. The secondary structure (*SS*) elements from the crystal structure of yDot1 and the predicted ones of aKMT4 are shown *below* and *above* the alignment, respectively. *Rectangles*, α helices; *arrows*, β strands. The conserved Dot1 motifs (*I, II, III*, etc.) are indicated *below* the yDot1 sequence as described (6, 43). The invariant or conserved residues are all in the catalytic domain and *shaded* in *black*. The species abbreviations with NCBI accession numbers and their aligned regions are as follows: *Sis*(YP_005648729)/3–161, *S. islandicus* REY; *HS*(Q8TEK3)/128–326, *Homo sapiens*; *MM*(EDL31504)/137–335, *Mus musculus*; *DR*(XP_001919781)/137–335, *Danio rerio*; *DM*(Q8INR6)/134–332, *Drosophila melanogaster*; *CE*(CCD69916.1)/165–365, *Caenorhabditis elegans*; *SC*(Q04089)/364–564, *S. cerevisiae*. *C*, three-dimensional model confirms structural compatibility of archaeal KMT4 with the Dot1 protein family. The x-ray crystal structure of yDot1 (Protein Data Bank code 1U2Z) was used to compare with the predicted Sul7d of aKMT4.
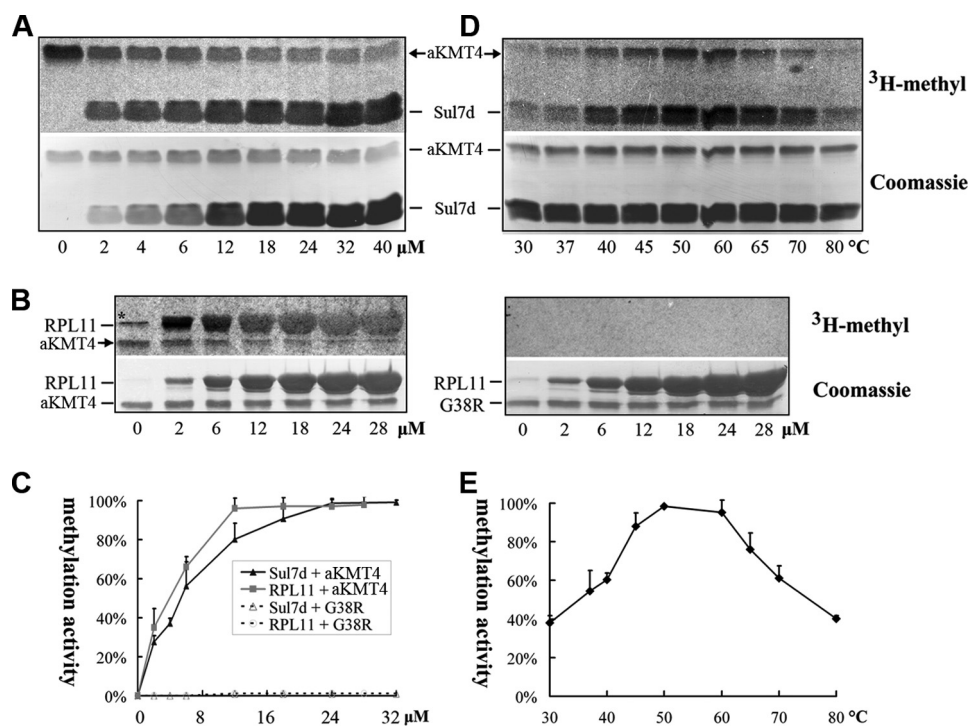
FIGURE 2. **aKMT4 possesses robust methylation activity on Sul7d and RPL11 *in vitro*.** The MTase transfers a [3H]-methyl group to the protein substrates from [3H]AdoMet as the methyl donor. Either wild type aKMT4 or *aKMT4*-G38R mutant (2 μM) was incubated with increasing amounts of Sul7d (*A*) or RPL11 (*B*) in a total volume of 30 μl at 70 °C for 3 h. The molar concentration of substrates is indicated. Proteins were separated by 15% SDS-PAGE for 2 h and transferred to a PVDF membrane. [3H]-Methyl incorporation in each protein band was visualized by autoradiography (*A*, *B*, and *D*) or quantified by scintillation counting (*C* and *E*). The amounts of enzymes and substrates were evidenced by Coomassie staining. aKMT4 migrates in the gel with an apparent mass of 18 kDa. *A*, methyltransferase activity on Sul7d was visualized by autoradiography. *B*, RPL11 was methylated by aKMT4 but not by the G38R mutant enzyme. The *asterisk* denotes a contaminant protein probably from *E. coli*, which is also methylated by aKMT4. *C*, quantification of the tritium incorporation into Sul7d and RPL11 by scintillation counting. aKMT4 transfers the methyl group to Sul7d and RPL11 with similar efficiency. *D*, aKMT4 remains active on Sul7d and RPL11 in a broad range of temperatures with optimal T_m between 50 and 60 °C *in vitro*. *E*, quantification of *D* by scintillation counting. Averages from at least three independent experiments ± S.D. (*error bars*) are shown.

tion mixture was directly spotted on Whatman P-81 filter paper right at the end of the reaction. P-81 paper was then washed four times with 50 mM NaHCO₃ to remove free [3H]AdoMet, and the incorporation of [3H]-methyl was monitored through a liquid scintillation counter. This direct counting approach was abandoned after we noticed that aKMT4 possesses automethylation activity. The methyltransferase activity presented in this study was measured through two different procedures. First, proteins from the methylation reaction were separated through 15% SDS-PAGE for about 2 h and transferred to a PVDF membrane using Bio-Rad Transblot. The membrane was stained by Coomassie Brilliant Blue R250. Autoradiography was carried out using a high speed gadolinium oxysulfide intensifying screen (Yuhuan Medical Instrument Co., Zhejiang, China). Next, each substrate protein band was cut from the membrane, and then scintillation counting was used to quantify the [3H] cpm. Background levels were obtained from the lanes in the absence of the targets. The value obtained from scintillation counting was corrected for background. The band of aKMT4 itself was also cut and counted for automethylation activity. For analysis of optimum pH, 50 mM Hepes or 50 mM Tris-HCl was used to obtain the pH gradient. All experiments were repeated at least three times. For quantification assays, averages from at least three independent measures and S.D. values were calculated.

Mass Spectrometry—The methylated sites were determined by MS/MS. Recombinant proteins (aKMT4, Sul7d, or Cren7) or

purified native Sul7d bands were sliced and in-gel digested with trypsin or chymotrypsin. Tryptic fragments were analyzed by Orbitrap Velos (Thermo Scientific) as described (40).

DNA Binding Assays by Gel Shift—The gel shift assays were carried out as described (25) with minor modifications. Increasing amounts of aKMT4 protein were incubated with each kind of 5′-labeled DNA or RNA for 30 min in a 10-μl volume containing 50 mM Tris-HCl, 30 mM NaCl, 10% glycerol, 1 mM EDTA, 2 mM DTT, 0.25 mg/ml BSA. The samples were analyzed through a 5% native polyacrylamide gel.

DNA Stimulation and Kinetic Assays—To simulate the *in vivo* environment of chromatin proteins, 50 pmol of salmon sperm DNA (sheared to 0.5–5 kb) or denatured DNA was preincubated with the enzyme and [3H]AdoMet at 4 °C for 25 min. The methylation reaction was then started by the addition of purified substrates and continued for 1 h at 50 °C. To clarify if chromatin proteins were methylated before or after they associated with DNA, DNA was added into reactions through three different combinations of preincubation: [(*E* +DNA) + Sul7d], [(*E* +Sul7d) + DNA], and [(Sul7d + DNA) + *E*)]. For kinetic analysis, different concentrations of Sul7d were incubated with DNA prior to the standard [3H]-methyl incorporation reactions for 1.5 h, or 10 μM Sul7d was subjected to standard reactions for the different time points. The apparent K_m and V_{max} values of Sul7d methylation by aKMT4 were calculated by a standard Michaelis-Menten equation.

RESULTS

Dot1-like Proteins Are Widespread in Crenarchaea and Some Euryarchaea—To explore whether there are Dot1 orthologs in prokaryotes, we carried out a PSI-BLAST search in bacterial and archaeal genome database using exceptionally highly conserved motifs (I, II, and III as shown in Fig. 1). Putative Dot1 orthologs were retrieved with statistically significant *E* values from *Sulfolobus* genomes (41, 42). Among them, a putative ribosomal protein L11 methyltransferase annotated in *Sulfolobus* genomes (NCBI entry YP_005648729, ORF *SiRe-1449* as in *S. islandicus* REY15A, hereafter referred to as aKMT4 (archaeal lysine methyltransferase 4)) showed relatively high similarity to Dot1 from *Saccharomyces cerevisiae*. Unlike its eukaryotic orthologs, however, archaeal Dot1-like proteins completely lack the substrate recognition domain and only comprise the typical Dot1 family methyltransferase catalytic core (Fig. 1*A*). The main region of sequence similarity lies in the Gly-rich AdoMet binding motif I (DX_3DLGXGXG), motif II (GXE), motif III (F(L/V/F)), motif VI (GX(R/K)(V/I)(V/I)S), and motif X (YXXT) (Fig. 1*B*). Despite the low overall sequence identity between aKMT4 and its eukaryotic orthologs, the predicted secondary structure of aKMT4 matched more than 80% of the secondary structure assignments of yDot1 (compare the schematics *above* and *below* the alignment in Fig. 1*B*). The predicted structure of aKMT4 consists of a seven-stranded β-sheet, which is a characteristic feature of the Dot1 family (PF08123 in the PFAM database) (6, 43). A three-dimensional model of aKMT4 was generated by comparative modeling based on alignment from Fig. 1*B* and the crystal structure of yDot1 (33) (Fig. 1*C*). The aKMT4 model is compatible with the structural conservation between the prokaryotic and eukaryotic members of the Dot1 family.

A BLAST search showed that aKMT4 was widely distributed in all crenarchaeal lineages and some euryarchaeal lineages (supplemental Fig. S1). Besides aKMT4, many of these archaeal lineages encode a histone deacetylase Sir2 homolog, which is believed to play a similar role to its eukaryotic homolog in transcription silencing through deacetylation of Alba (Sso10b) (44). The phylogenetic results indicate that archaeal KMT4 might represent a prototype of this protein family and an evolutionary precursor to eukaryotic Dot1 proteins.

aKMT4 Possesses Intrinsic MTase Activity under Various Conditions—Because Sul7d constitutes the main scaffold of chromatin and has been shown to be methylated across *Sulfolobus* species (19, 23, 24), the recombinant protein expressed in *E. coli* was used as a prospective substrate to directly test if aKMT4 has intrinsic MTase activity. Increased covalent ³H-methyl-Sul7d products were clearly visualized by autoradiography on the PVDF membrane after separation by SDS-PAGE when recombinant aKMT4 was incubated with the increasing amounts of substrate and [³H]AdoMet supplied as the methyl donor (Fig. 2*A*). Next, we examined whether aKMT4 is able to act on putative substrate ribosomal protein L11. Tritium incorporation into RPL11 was also detected (Fig. 2*B*) at a level comparable with that of Sul7d. The optimal ratio of substrate to enzyme is ~6. The apparent K_m values of both substrates are ~4 μM, indicating a very weak association
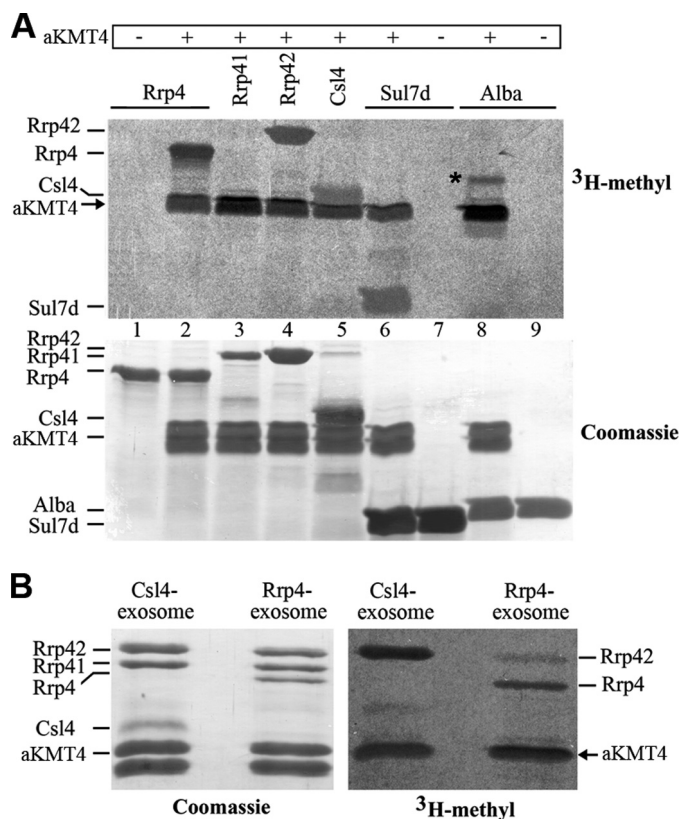

FIGURE 3. **aKMT4 acts on two RNA exosome complexes in different pattern.** Standard protein methylation reactions were conducted as described under "Experimental Procedures." Various putative substrates were incubated with 8 μM enzyme at 50 °C for 3 h. *A*, all RNA exosome subunits except for catalytic subunit Rrp41 can be methylated *in vitro*. Note that Alba, the most conserved nucleic acid binding protein in Archaea, has no methylated product. The *asterisk* denotes a nonspecific protein that can be methylated by aKMT4. *B*, heterogeneous complexes of RNA exosome proteins are differently methylated by aKMT4. Two exosome complexes, Rrp4-exosome (Rrp42/41/Rrp4) and Csl4-exosome (Rrp42/41/Csl4), were reconstituted *in vitro* using purified recombinant proteins, as described by Witharana *et al.* (39). Note that the methylation of Rrp42 in Rrp4-exosome and Csl4 in Csl4-exosome was greatly reduced.

between aKMT4 and its substrates (Fig. 2*C*). Furthermore, no interaction between aKMT4 and its substrates (Sul7d or RPL11) could be detected by various approaches, such as GST pull-down, far Western, gel filtration, and coimmunoprecipitation with endogenous proteins (data not shown). These results prompted us to propose a "hit and run" model for aKMT4, which is consistent with the strict distributive pattern of eukaryotic Dot1 (45).

In all Dot1 family MTases, the key residues in binding of AdoMet are within the highly conserved DLGXGXG motif I (see *arrow* in Fig. 1*B*) (4, 6). When one of the invariant glycine residues, Gly-38, was changed to arginine, the MTase activity was completely abolished (Fig. 2, *B* and *C*), which is in consistent with its counterpart mutant, yeast *dot1*-G401R (5), indicating that the aKMT4 shares the same essential, functional motifs with the eukaryotic Dot1 family.

Considering that *Sulfolobus* cells thrive in high temperatures (optimal growth T_m = 80 °C), the activity of aKMT4 was examined under various conditions. Although aKMT4 sustained methylation activity within a broad range of temperature, to our surprise, the optimal temperature for the MTase was about
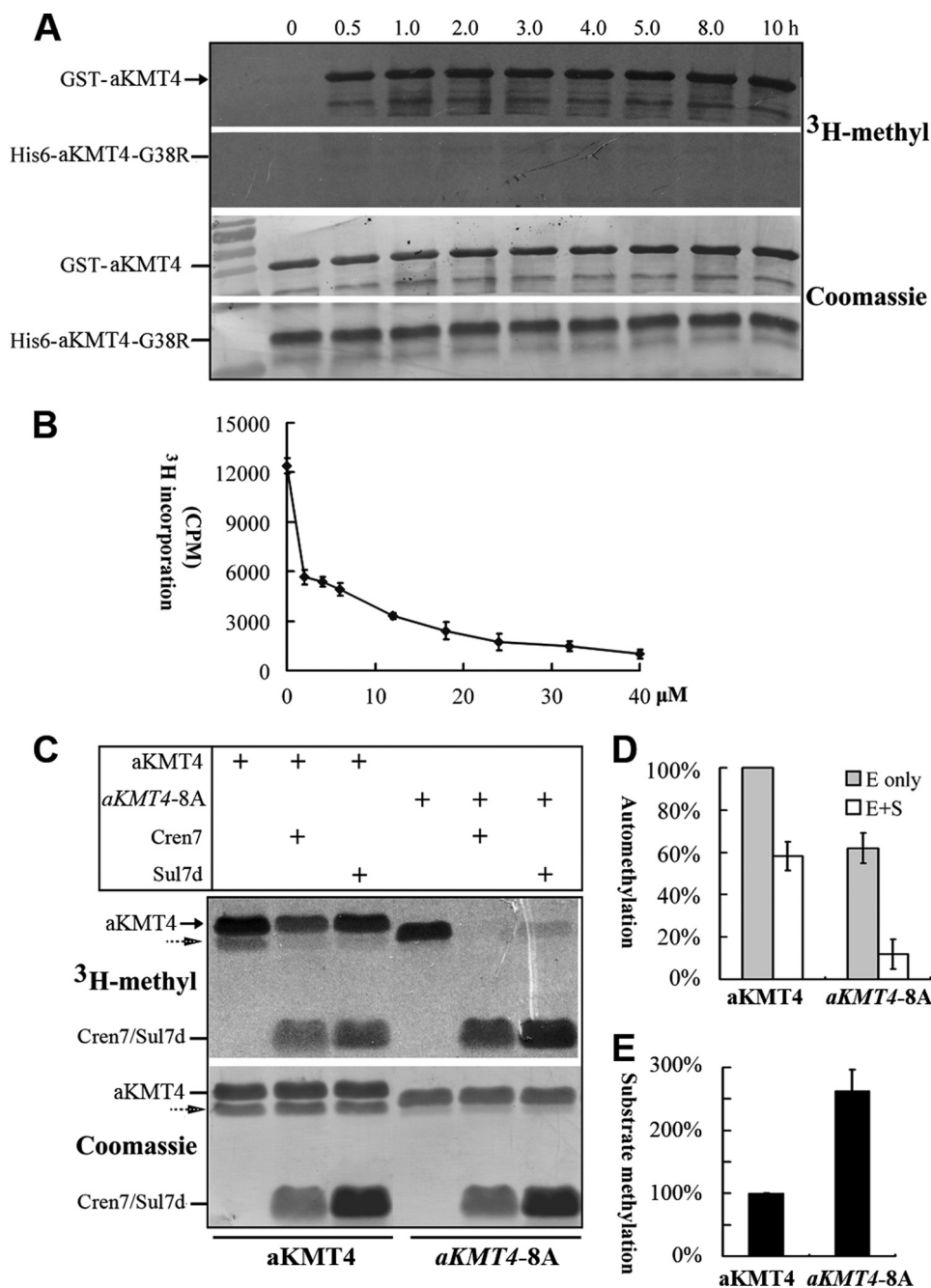
FIGURE 4. **aKMT4 can self-methylate and compete with substrate for the incoming methyl group.** *A*, automethylation occurs intramolecularly. 4 μM GST-tagged aKMT4 was incubated with 8 μM His$_6$-tagged *aKMT4*-G38R mutant at 50 °C for different times (shown in h) as indicated. Note that G38R mutant is not able to be methylated even after 10 h, whereas GST-tagged wild type enzyme can be automethylated within 0.5 h. *B*, substrates (Sul7d, Cren7, and RPL11) inhibit automethylation activity of aKMT4 in a concentration-dependent manner. Methylation reactions were carried out as described for Fig. 1, *A* and *B*. Tritium incorporation was quantified by scintillation counting. *C*, inhibition of automethylation leads to much more efficient methyl transfer to suitable substrates. *aKMT4*-8A enzymes (containing eight alanine substitutions of lysine sites near N and C termini) significantly increase the methylation levels on the other proteins with abolished automethylation activity. 4 μM wild type or *aKMT4*-8A mutant enzymes were incubated with a 12 μM concentration of each substrate (Cren7, Sul7d, or RPL11) at 50 °C for 3 h. Shown are quantifications of automethylation (*D*) and substrate methylation (*E*) levels from *C*. *Error bars*, S.D.

50–60 °C (Fig. 2, *D* and *E*), presumably due to the compromised protein stability at high temperature under *in vitro* conditions. aKMT4 was active over a wide range of pH with maximum activity around pH 8–9 (Data not shown). This is a characteristic of Dot1 family enzymes and it is distinct from SET domain KMTs, which require an unusually high pH optimum ($\sim$10) to ensure that the amino group of the target lysine becomes partially deprotonated (6). Taken together, we conclude that Crenarchaea encode a conserved AdoMet-dependent KMT4/Dot1 family MTase, which methylates both chromatin and ribosomal proteins. Archaeal Dot1 shares common active sites and structural and biochemical characteristics similar to those of the eukaryotic Dot1 family.

Heterogeneous Complexes of the RNA Exosome Are Methylated in Different Patterns—Considering the complete lack of the substrate recognition domain, as shown in Fig. 1, we spec-

TABLE 1

Methylated lysine residues identified by MS/MS in self-methylated aKMT4, recombinant Sul7d methylated by aKMT4 *in vitro,* and native Sul7d

Substrates	Potential methylated lysine residues shown in bold*
aKMT4	MSYVPHVPYV PTPEKVVRRM LEIA**K**VSQDD IVYDLGCGDG RIIITAA**K**DF NV**KK**AVGVEI NDERIREALA NIE**K**NGVTGR ASIVKGNFFE VDISEATVVT MFLLTNVNEM LKPKLE**K**EL**K** PGTRVVSHEF EIRGWNP**K**EV I**K**VEDGNMNH TVYLYVIGEH K
Native Sul7d	MTTV**K**F**K**YKG EE**K**QVDTSKI KKVWRVGKMI SFTYDEGGGK TGRGAVSE**K**D AP**K**ELLQMLE **K**Q**KK**LE
Sul7d	MTTV**K**F**K**Y**K**G EE**K**QVDTSKI KKVWRVG**K**MI SFTYDEGGG**K** TGRGAVSE**K**D AP**K**ELLQMLE **K**Q**KK**LE
sul7d-5A	MTTVAFAY**K**G EE**K**QVDTSKI KKVWRVG**K**MI SFTYDEGGG**K** TGRGAVSE**K**D AP**K**ELLQMLE AQAALE
Sul7d with DNA	MTTV**K**F**K**YKG EE**K**QVDTSKI KKVWRVGKMI SFTYDEGGGK TGRGAVSE**K**D AP**K**ELLQMLE **K**Q**KK**LE
sul7d-5A with DNA	MTTVAFAYKG EE**K**QVDTSKI KKVWRVGKMI SFTYDEGGGK TGRGAVSE**K**D AP**K**ELLQMLE AQAALE

*, All lysines in boldface type were identified to exist in both monomethylated and unmethylated forms.

ulated that aKMT4 might act on a diverse set of substrates. Besides Sul7d and RPL11, a variety of other proteins from *Sulfolobus* could be methylated by the aKMT4 enzyme (Fig. 3*A*). Chromatin protein Cren7, coexisting with aKMT4 throughout the Crenarchaea branch, was methylated to a similar extent as Sul7d and RPL11 *in vitro.* In contrast, Alba/Sso10b and *SiRE_1713* did not serve as substrates, although all of them contain a considerable number of lysine residues (Fig. 3*A*). No methylated form of Alba/Sso10b and *SiRE_1713* has been reported in *Sulfolobus* cells either. Thus, unlike its eukaryotic homolog, which targets strictly to histone H3, aKMT4 can act on a set of versatile substrates.

Next we assessed how aKMT4 acts on multisubunit protein complexes. One of the best studied complexes in *Sulfolobus* is the RNA exosome, which is responsible for RNA processing and degradation (39, 46). The archaeal exosome consists of only four proteins with structurally similarity to the eukaryotic exosome core. The catalytically active Rrp41 and its structural partner Rrp42 form a hexameric ring. Rrp4 (or Csl4) forms a trimeric cap on one side of the ring for RNA substrate binding (39). Taking advantage of the existence of heterogeneous complexes of the exosome in *Sulfolobus*, we examined how aKMT4 works on protein complex substrates. The two forms of exosome complex, called Rrp4-exosome and Csl4-exosome, were reconstituted *in vitro* using purified recombinant proteins (39). Intriguingly, when they were subjected to MTase assays alongside each individual subunit, different methylation patterns could be seen (Fig. 3). Rrp4, Rrp42, and Csl4, but not the catalytic subunit Rrp41, were methylated by aKMT4 *in vitro* (Fig. 3*A*, *lane 3*). However, Csl4 methylation disappeared in the

reconstituted Csl4-exosome (Fig. 3, compare *Csl4-exosome lanes* in *B* with *lane 5* in *A*). Methylation of Rrp42 was retained in Csl4-exosome but was nearly eliminated in Rrp4-exosome (Fig. 3*B*, compare *Csl4-exosome* and *Rrp4-exosome lanes*). Comparison of the crystal structure of these two complexes reveals that the methylation sites of the Rrp42 subunit might be masked by the relatively large Rrp4 cap (Protein Data Bank code 2JE6) but remain accessible in the Csl4-exosome (Protein Data Bank code 2BA1) (46, 47). These *in vitro* results hint at a possible role of aKMT4 in regulation of RNA substrate recognition and processing through differential methylation of RNA exosome complexes.

aKMT4 Can Self-methylate Intramolecularly and Compete with Substrates for Methyl Group—During all methylation assays by tritium autoradiography, it is worth noting that there was a second methylated band whose mobility corresponds to the aKMT4 band in the Coomassie-stained gel (see Figs. 2 and 3, aKMT4 labeled with an *arrow*). No ^{3}H-methyl band can be detected in the substrate-only reactions, which ruled out the possibility that there were contaminating MTase activity in the purified proteins from *E. coli* (Fig. 3*A*, *lanes 1*, *7*, and *9*). Furthermore, this ^{3}H-methyl band remained in the absence of substrates and disappeared if the aKMT4 enzyme was replaced by G38R mutant (Fig. 4*A*). These results suggest that aKMT4 enzyme can methylate itself. When an excess amount of the His$_6$-tagged *aKMT4*-G38R mutant protein was used as substrate, ^{3}H-methyl could only be incorporated into GST-tagged aKMT4 wild type protein and not into *aKMT4*-G38R mutant even after a 10-h reaction (Fig. 4*A*). We consider it unlikely that the Gly-38 residue is the automethylation site of the enzyme.
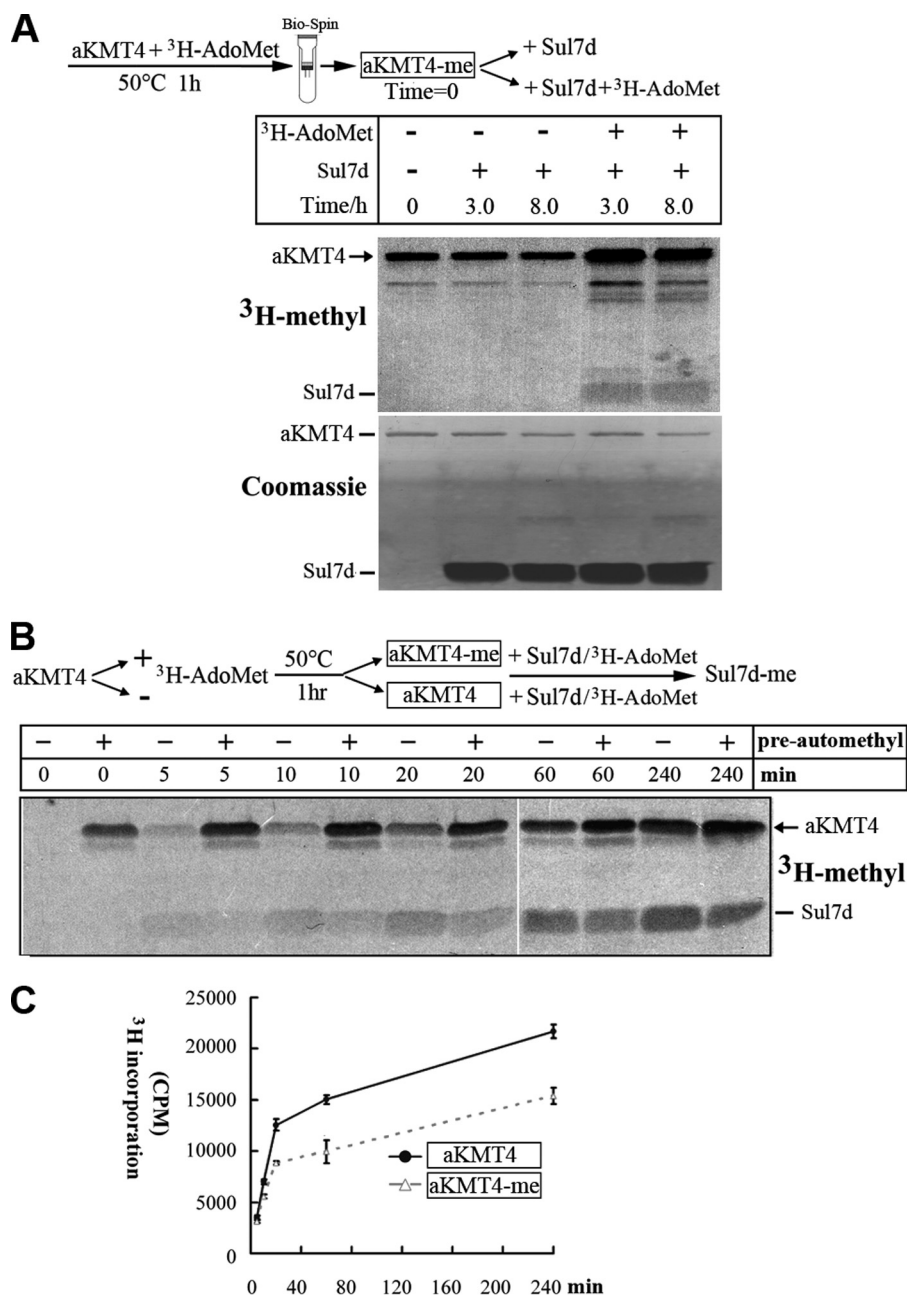
FIGURE 5. **Self-methylated aKMT4 cannot transfer its methyl group to Sul7d.** *A*, 4 μM GST-aKMT4 was self-methylated after incubation with [³H]AdoMet at 50 °C for 1 h. Unincorporated [³H]AdoMet was removed through a Bio-Spin P30 column (Bio-Rad). An excess amount of Sul7d (12 μM) was then added to self-methylated aKMT4 in the presence (+) or absence (−) of [³H]AdoMet at 50 °C for 3 or 8 h. Methylation of Sul7d was detected by autoradiography. *B*, self-methylated aKMT4 shows a relatively slow rate of substrate methylation. The methylation kinetics of Sul7d were monitored at different time points, as indicated *above* each *lane* after preincubation of aKMT4 in the presence (+) or absence (−) of [³H]AdoMet (as control) at 50 °C for 1 h. *C*, quantification counts of tritium incorporation into Sul7d from *B* were plotted against reaction time. Averages from at least three independent experiments ± S.D. (*error bars*) are shown.

This indicates that automethylation occurs intramolecularly near the AdoMet binding or the active sites of the enzyme.

Furthermore, the intensity of the self-methylated band was remarkably reduced in the presence of proper substrates, such as Sul7d, RPL11, and Cren7. These substrates inhibited automethylation of aKMT4 in a concentration-dependent manner (Figs. 2 (*A* and *B*) and 4*B*). This suggests that aKMT4 transfers a methyl group from incoming AdoMet to itself in the absence of substrates. When the enzyme associates with the proper substrates, however, the enzyme prefers to transfer the methyl group to the substrates rather than to itself. We also noticed that a terminal truncated fragment of aKMT4 caused by non-specific thrombin digestion of GST-tagged Dot1 showed abrogated automethylation, particularly in the presence of substrates (see *dashed arrow* in Fig. 4*C*). The AdoMet-binding and active sites are also near N and C ends (Fig. 1). Next, we mutated all eight lysine residues to alanine at both N and C termini and obtained *aKMT4*-8A protein. Automethylation of *aKMT4*-8A was almost completely removed in the presence of substrates, such as Cren7 and Sul7d (Fig. 4, *C* and *D*). Surprisingly,

aKMT4-8A mutant alone still retained significant automethylation activity in the absence of substrates (Fig. 4*C*), indicating that the enzyme has alternative automethylation sites. This turned out to be true by MS/MS analysis (Table 1), indicating that aKMT4 has low amino acid sequence specificity. Meanwhile, the activity of *aKMT4*-8A on these substrates increased more than 150% compared with wild type enzyme (Fig. 4, *C* and *E*). These results suggest that there is competition between aKMT4 and substrates for the incoming methyl group, and inhibition of automethylation significantly increases the methylation efficiency of substrates.

Self-methylated aKMT4 Is Not an Intermediate State for Methyl Transfer—To answer if methylated aKMT4 can transfer its methyl group to other substrates, we carried out a preautomethylation assay (Fig. 5*A*). The MTase was self-methylated (*aKMT4-me*) by preincubation with [³H]AdoMet. Unincorporated AdoMet was then removed through a Bio-Spin P30 column. Sul7d could hardly be methylated before [³H]AdoMet was added back (Fig. 5*A*). Actually, recombinant aKMT4 purified from *E. coli* was found to be partially self-methylated by MS/MS (Table 1). If Ado-Met was omitted in reactions, no methylation of Sul7d or other substrates by self-methylated aKMT4 was detected (data not shown). These results suggest that aKMT4 undergoes stable self-methylation and that the methylated aKMT4 molecule cannot transfer its methyl group to other molecules.

Next, we monitored the methylation kinetics of Sul7d after preincubation of aKMT4 in the presence (+) or absence (−) (as control) of [³H]AdoMet at 50 °C for 1 h (Fig. 5*B*). Self-methylated aKMT4 showed relatively attenuated activity on Sul7d substrates compared with aKMT4 without preautomethylation, although both of them were incubated at high temperature equally. Quantification by scintillation counting showed an about 30% slower reaction rate of self-methylated aKMT4 than aKMT4 (Fig. 5*C*). These data provide clues that automethylation may moderately inhibit activity of aKMT4.

aKMT4 Has Extremely Low Amino Acid Sequence Specificity—aKMT4 is not only able to transfer the methyl group to a broad range of proteins, including itself; the above mentioned results for the *aKMT4*-8A mutant also indicate that it has degenerate amino acid sequence bias. When we compared the context sequences of lysine residues in all known substrate and nonsubstrate proteins of aKMT4, there were no apparent conserved motifs. Native Cren7 has been identified to be monomethylated at up to five sites among its 12 lysine residues in *Sulfolobus* cells (28). In both native Cren7 and recombinant proteins catalyzed by aKMT4, some lysine sites, including Lys-16, -24, and -31, were more frequently identified as being monomethylated through MS/MS analysis (supplemental Fig. S2) (27). The correlation between *in vivo* and *in vitro* methylation data suggests that aKMT4 may serve as the main MTase for Cren7 methylation in *Sulfolobus*. Alanine scanning was next carried out to confirm the methylation sites of aKMT4 *in vitro*. aKMT4 showed almost the same activity on the single mutations of Lys-16, -24, or -31 in Cren7 (data not shown). Surprisingly, *cren7*-3A, *cren7*-5A, and even *cren7*-7A with seven lysine residues mutated showed only moderate reduction of methylation level (Fig. 6*A*). Cren7 methylation was completely abol-
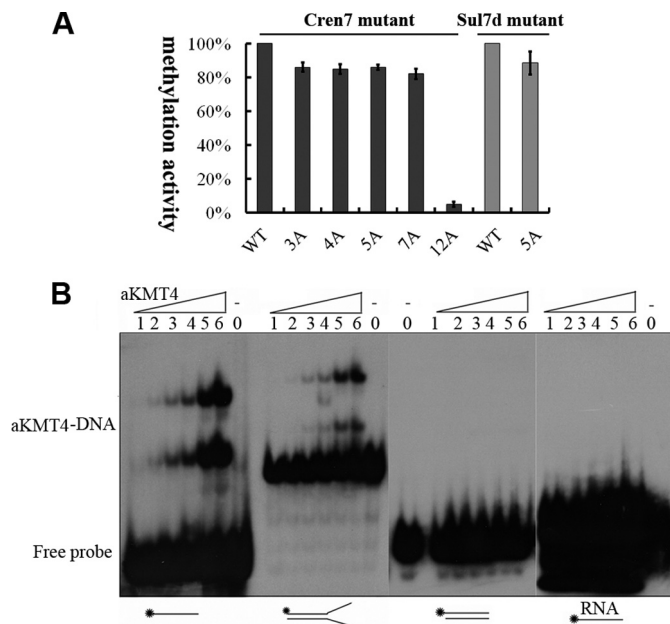

FIGURE 6. *A*, alanine scanning analysis demonstrated aKMT4 catalyzes methyl transfer to Cren7 or Sul7d with an extremely low amino acid sequence bias. Most of the mutants were efficiently methylated by aKMT4 *in vitro*, at a slightly lower level than WT. Mutants used were as follows: *cren7*-3A (Lys-16, -24, and -31); *cren7*-4A (Lys-16, -24, -31, and -42); *cren7*-5A (Lys-11, -16, -24, -31, and -42); *cren7*-7A (Lys-11, -16, -24, -31, -34, -42, and -53); *cren7*-12A (all 12 lysines); and *sul7d*-5A (Lys-5, -7, -61, -63, and -64). Averages from three independent experiments ± S.D. (*error bars*) were shown. *B*, aKMT4 can weakly bind to single-stranded DNA. Gel shift assays were carried out using aKMT4 and different kinds of nucleic acid probes. 100 fmol of ³²P-labeled single-stranded DNA, Y-shaped DNA, double-stranded DNA, or single-stranded RNA was incubated with increasing concentrations of aKMT4 protein at 0 °C for 30 min. The amount of aKMT4 used was as follows: 0.2 nmol (*lane 1*), 0.8 nmol (*lane 2*), 1.4 nmol (*lane 3*), 2.4 nmol (*lane 4*), 5 nmol (*lane 5*), and 7 nmol (*lane 6*).

ished when all 12 lysine residues were changed, indicating that aKMT4 is specific to lysine. These results suggest that aKMT4 is a promiscuous protein MTase with extremely low sequence bias, which is consistent with the variegated methylation pattern of Cren7 and a number of other proteins in *Sulfolobus* cells (28).

We noticed that another chromatin protein, Sul7d, was methylated *in vivo* most intensively near its tails (Lys-5 and -7 and Lys-61, -63, and -64) by MS/MS assays (Table 1), which is in agreement with previous reports (19, 23, 24). Meanwhile, Lys-53 and Lys-49 were also detected to be methylated in native Sul7d, although to a relatively lower extent (Table 1), which is probably due to strain difference and variegated methylation pattern in *Sulfolobus*. Surprisingly, recombinant Sul7d was found to be methylated by aKMT4 *in vitro* at most, if not all, of the lysine residues, which is very similar to the above mentioned Cren7 methylation pattern (Table 1). Alanine scanning showed that *sul7d*-5A (Lys-5,-7, -61, -63, and -64) was methylated to a similar extent as wild type (Fig. 6*A*). MS/MS data showed that *sul7d*-5A retains all potential aKMT4 target sites of Sul7d except the substituted lysines (Table 1). The discrepancy between the *in vitro* and *in vivo* methylation patterns of Sul7d implies that aKMT4 could be regulated *in vivo*, although it is a promiscuous enzyme *per se*.

DNA Stimulates the Activity of aKMT4 on Sul7d but Not Cren7—Both yDot1 and hDot1L contain a lysine-rich DNA binding domain, which helps to target itself to nucleosomes and
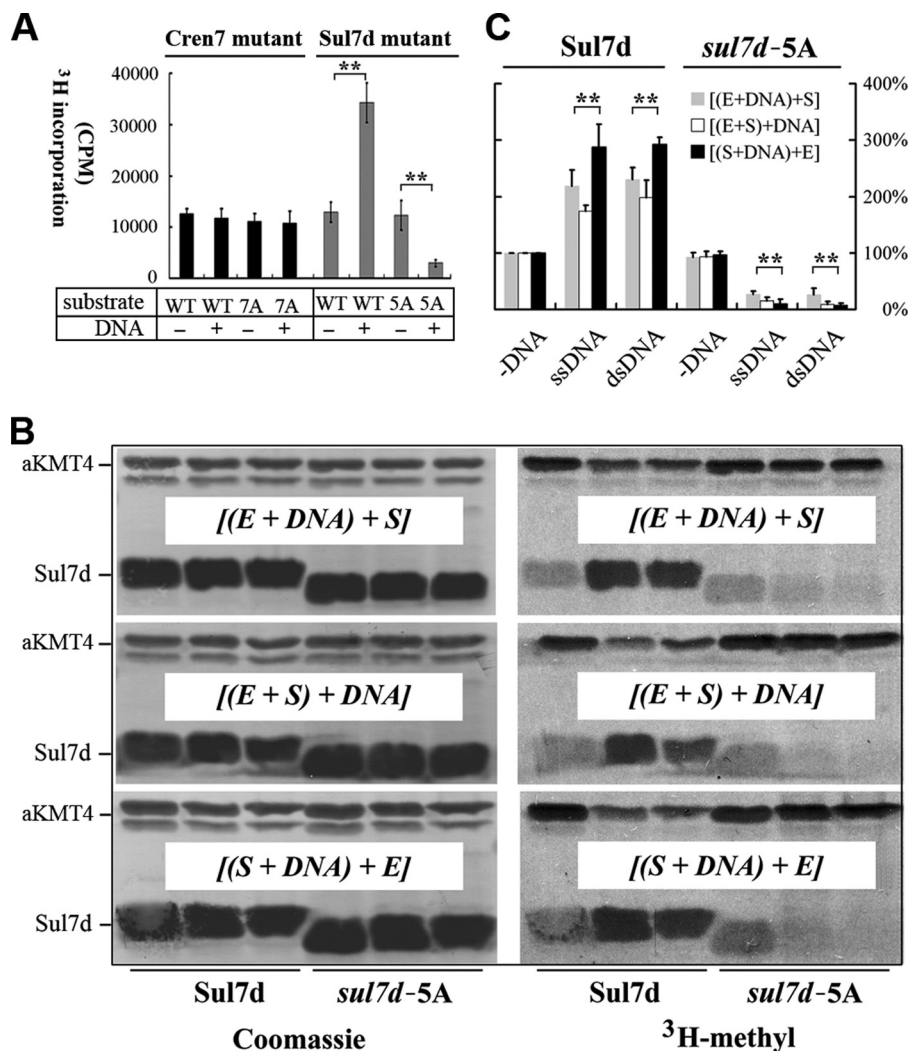
FIGURE 7. **aKMT4 methylates the main chromatin component Sul7d in the context of chromatin.** Sul7d and *sul7d*-5A mutant proteins were subjected to standard methylation reactions by aKMT4 in the presence or absence of DNA. *A*, methylation of Sul7d, but not Cren7, was significantly affected by DNA. *B*, the methylation of *sul7d*-5A is inhibited in the presence of either ssDNA or dsDNA, whereas the efficiency of wild type Sul7d methylation is raised by DNA to about 3-fold. Meanwhile, the intensity of automethylation bands is significantly weakened. Such effects can be further enhanced by preincubation of Sul7d and DNA prior to the addition of the enzyme ((Sul7d + DNA) + *E*). Three different incubation orders are indicated as [(*E* + DNA) + Sul7d], [(*E* + Sul7d) + DNA], and [(Sul7d + DNA) + *E*]. *C*, quantification of the substrate protein bands in *B* by scintillation counting from three different repeats with S.D. values (*error bars*). **, $p < 0.01$, significant differences between [(Sul7d + DNA) + *E*] and [(*E* + DNA) + Sul7d].

is critical for enzyme activity (4, 6). Gel shift demonstrated that aKMT4 is able to associate very weakly with single-stranded DNA and forklike structured DNA but not double-stranded DNA or RNA (Fig. 6*B*). The enzyme-DNA complexes showed multiple shift bands, indicating that more than one aKMT4 molecule can bind to the same DNA oligomer. Transient single-stranded regions are likely to form more extensively in genomic DNA under the high growth temperature of *Sulfolobus* cells. The ability to bind single-stranded DNA regions might contribute to the performance of aKMT4 in the genomic context.

To test this hypothesis, we examined the effect of DNA on the MTase activity of aKMT4. The addition of DNA had almost no effect on methylation of Cren7 or *cren7*-7A by aKMT4, indicating that the methylation of Cren7 is not affected by DNA (Fig. 7*A*). On the contrary, the tritium incorporation into Sul7d increased by nearly 2-fold in the presence of DNA (Fig. 7*A*). Both native (dsDNA) and dena-

tured (ssDNA) DNA showed a similar stimulatory effect (Fig. 7, *B* and *C*). Although Sul7d is not able to bind ssDNA, the denatured ssDNA might partially reanneal at the 50 °C reaction condition. Furthermore, as a robust DNA-binding protein, Sul7d is capable of stabilizing the duplex DNA at high temperatures (37, 48). This result illustrates that aKMT4 has a higher efficiency for the transfer of the methyl group to the Sul7d proteins bound to DNA.

The *sul7d*-5A mutant has been identified to be methylated by aKMT4 *in vitro* at a level comparable with WT (Fig. 6*A*). Intriguingly, methylation of *sul7d*-5A protein was significantly inhibited in the presence of DNA (Fig. 7, *A–C*), suggesting that *in vitro* methylation of *sul7d*-5A alone resulted from the extra exposed sites, most likely Lys-28 and other lysines (Table 1) located near the protein-DNA interface (Val-26 and Met-29) (49). These results suggest that although aKMT4 has very low sequence bias, its specificity can nevertheless be influenced by the local structure of the target residues.
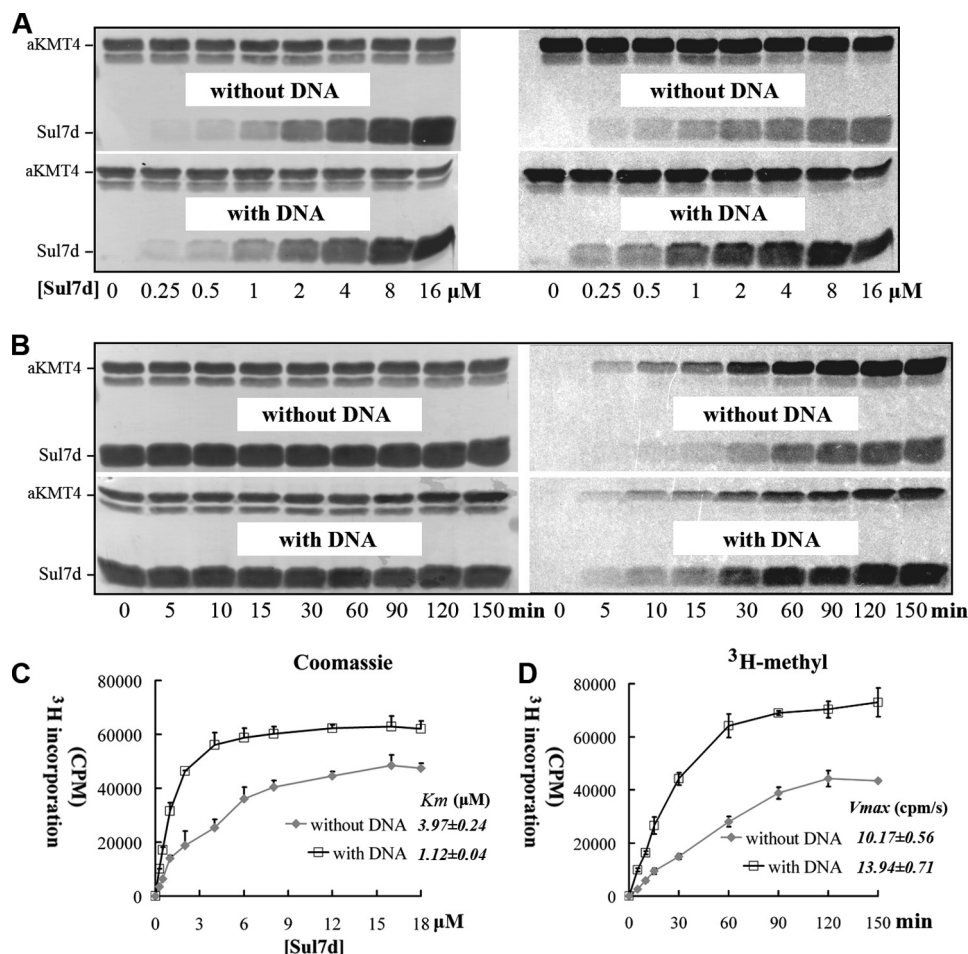
FIGURE 8. **Sul7d methylation by aKMT4 is significantly stimulated by DNA.** Protein methylation reactions were carried out essentially as in Fig. 7 with the reaction time extended from 1 to 1.5 h. The reactions with DNA were carried out as [(Sul7d + DNA) + E]. *A*, DNA increases Sul7d association with the MTase. Different amounts of Sul7d were incubated with 2 μM aKMT4 enzyme with or without DNA at 50 °C for 1.5 h. *B*, DNA increases the methyl transfer rate of aKMT4. 10 μM Sul7d was subjected to methylation reaction with 2 μM aKMT4 in the presence or absence of DNA at 50 °C. Reactions were quenched at different time points, and methyl incorporation was quantified and plotted against time. *C* and *D*, quantification of ^{3}H-methylation of Sul7d in *A* and *B*, respectively. The data represent the mean ± S.D. (*error bars*) from three independent experiments.

aKMT4 Catalyzes Methylation of Sul7d in the Chromatin Context—The fact that Sul7d is exclusively methylated at the free N and C tails in *Sulfolobus* cells (Table 1) (23, 24) implies that Sul7d should only be methylated in the chromatin context *in vivo*. This hypothesis was further tested by changing the order of addition of DNA to MTase and substrates (Fig. 7, *B* and *C*). When *sul7d*-5A was incubated with DNA prior to the addition of aKMT4, methylation was barely detectable. Meanwhile, the methylation level of Sul7d increased much more significantly if Sul7d was incubated with DNA first (S + DNA) (Fig. 7, *B* and *C*). Preincubation of the enzyme and DNA (E + DNA) also showed a moderately stronger stimulation effect than the addition of DNA to the premix of enzyme and Sul7d (E + Sul7d).

Kinetic analysis of Sul7d methylation provided further evidence to support this conclusion (Fig. 8). The apparent K_m value for Sul7d substrate was reduced from 3.97 ± 0.24 μM in the absence of DNA to about 1.12 ± 0.04 μM in the presence of DNA (Fig. 8, *A* and *C*). Meanwhile, aKMT4 catalyzed ^{3}H-methyl incorporation into DNA-associated Sul7d at an ~37% faster rate than into the free Sul7d (Fig. 8, *B* and *D*). These data suggest that DNA increases opportunities for Sul7d

to be hit and methylated by aKMT4. The overall increased methylation efficiency observed in the Sul7d-DNA complex mainly results from the increased local concentration of Sul7d molecules after forming the compact chromatin structure.

DISCUSSION

As much as we know about histone modifications in eukaryotes, our knowledge of the origins of the epigenetic regulation pathway and the evolution of the participating enzymes is still very limited. To address the possible evolutionary origin of histone modification enzymes, we identified and characterized a KMT4/Dot1-like methyltransferase from *S. islandicus*, which belongs to the third domain of life, Archaea, a closer lineage of Eukarya than Bacteria (16).

First, we showed that KMT4/Dot1-like genes are conserved, especially among Crenarchaea, whereas SET domain MTases only exist in a few methanogenic *Methanosarcina* species (Göl-SET) (26). Based on molecular evolutionary analysis, Aravind *et al.* (50) proposed that eukaryotic histone methylation systems might originate in bacteria, where these enzymes are utilized for synthesis of secondary metabolites, such as antibiotics and toxins. All lysine demethylase *KDM1* genes are also found to

share a common single origin from a bacterial amine oxidase *AOD* gene (51). Previously, Bell *et al.* suggested that acetylation/deacetylation of Alba might represent a primitive model for chromatin regulation analogous to histone modification in eukaryotes (44). It is possible that interplay between KMT4 and Sir2 participates in chromatin regulation in Archaea as in Eukarya. Our study provides experimental evidence to support the archaeal origin model of eukaryotic histone MTases and further suggests that aKMT4 family members might participate in regulation of chromatin structure and function in Archaea, just as their eukaryotic counterparts.

Second, aKMT4 catalyzes methylation of Sul7d and Cren7 in different patterns, although they are both believed to be main chromatin proteins in *Sulfolobus*. Methylation of Sul7d, but not Cren7, is significantly enhanced in the presence of DNA. It can be explained if Cren7 methylation occurs prior to DNA binding or the majority of methylation sites are not affected by the conformation change of Cren7 upon association with chromatin. DNA-associated Sul7d is found to be only methylated by aKMT4 *in vitro* at its free tails, which correlates well with the methylated sites of native Sul7d *in vivo* (19, 23, 24). These findings suggest that Sul7d is truly methylated only in the chromatin form *in vivo*. Despite its low sequence bias, the substrate recognition of aKMT4 might be regulated by the local chromatin environment and its DNA binding ability.

Third, another interesting finding is that aKMT4 undergoes automethylation intramolecularly *in vitro* and that the self-methylated form shows relatively compromised activity. Furthermore, automethylation can be inhibited in the presence of suitable substrates or DNA, which in turn increases the efficiency of methyl transfer to the substrates. Such properties of automethylation may provide a novel mechanism for regulation of MTase activity. Automethylation has not been observed for any other protein MTases except for PRMT10 from *Arabidopsis thaliana*. atPRMT10 can be automethylated at residue Arg-13 in the absence of suitable substrates *in vitro*, but, unlike aKMT4, automethylation does not seem to alter atPRMT10 activity, because the R13K mutation does not affect the ability of atPRMT10 to methylate histones (52). Some DNA methyltransferases, including mammalian Dnmt3a and bacterial Ada, are also automethylated. In this case, automethylation occurs on a cysteine in the active site, resulting in inactivation of the enzyme (53).

In bacteria, lysine methylation is limited to EF-Tu, ribosomal, and flagellar proteins (15). In eukarya, lysine methylation is catalyzed by sequence-specific MTases that typically have only a single target (3, 6). SET from *Methanosarcina* also methylates a single Lys-37 in the euryarchaeal chromatin protein MC1-α (26). However, lysine methylation is prevalent in Crenarchaea within a number of proteins bearing variegated methylation at multiple sites. For example, 21 methyl-lysines have been identified from nine subunits of the RNA polymerase complex purified from *Sulfolobus solfataricus* (28). aKMT4, also described by Chu *et al.* (27), has the properties of the MTase speculated to exist by Botting *et al.* (28). Most of the class I MTases are known to act on nucleic acid or chromatin proteins to regulate DNA metabolic processes, such as gene expression, RNA processing, and DNA repair (3, 4, 6). It is worth noting that aKMT4 is capable of catalyzing the methylation of a series of proteins, including chromatin proteins (Sul7d and Cren7), RNA exosome complexes, and ribosomal proteins. Although promiscuous methylation could be an artificial outcome of the *in vitro* conditions, our work suggests that versatile substrate specificity of aKMT4 might allow it to perform multiple roles in nucleic acid metabolism processes in Archaea. It will be of great interest to address the physiological function of aKMT4 and the methylation of its substrates in genetically tractable organisms in the future.

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